Biochemical Pharmacology, Vol. 40, No. 3, pp. 447–456, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

DIFFERENTIAL INHIBITION OF HISTAMINE RELEASE FROM MAST CELLS BY PROTEIN KINASE C INHIBITORS: STAUROSPORINE AND K-252a

JOHN R. WHITE,* DENISE ZEMBRYKI, NABIL HANNA and SEYMOUR MONG
Department of Immunology, Smith Kline & French Laboratories, King of Prussia, PA 19406-0939, U.S.A.

(*Received* 13 *July* 1989; *accepted* 8 *December* 1989)

Abstract—Pretreatment of rat peritoneal mast cells with either staurosporine or an analog K-252a [(8*R**, 9*S**,11*S**)-(−)-9-hydroxyl-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11-atriazadibenzo-[a,g]cycloocta[cde]trinden-1-one] led to a concentration-related inhibition of histamine release when the cells were stimulated with anti-IgE (IC_{50}: staurosporine = 110 nM; K-252a = 100 nM). In contrast, the two protein kinase C (PKC) inhibitors (1–1000 nM) partially (<15%) inhibited histamine release induced by compound 48/80 (0.5 to 1 μg/mL). Furthermore, prostaglandin E_2 (PGE_2) synthesis mediated by anti-IgE from rat peritoneal mast cells was also inhibited by staurosporine and K-252a (IC_{50} = 100 nM). Exposure of anti-arsenate IgE (anti-Ars-IgE) sensitized mouse bone marrow derived mast cells to arsenate-bovine serum albumin (Ars-BSA) led to the release of both histamine (510 ± 12.6 ng/10^6 cells) and immunoreactive leukotriene C_4 (LTC_4) (27.0 ± 2.6 ng/10^6 cells). Both histamine and LTC_4 release were inhibited by staurosporine and K-252a with an IC_{50} of 50 nM for both compounds. We also characterized a 45K molecular weight protein which is phosphorylated by PKC after Ars-BSA or phorbol, 12-myristate, 13-acetate (PMA) stimulation. This protein is phosphorylated in a broken cell preparation in which PKC is activated by phosphatidylserine/Diolein and Ca^{2+}. Peptide mapping by V8 protease of the phosphorylated 45K protein revealed that the 45K protein phosphorylation patterns induced by IgE or PMA or in the broken cell preparation are identical. Pretreatment of ^{32}P-labeled mouse bone marrow derived mast cells with either staurosporine or K-252a led to a concentration-related inhibition of 45K protein phosphorylation induced by PMA or Ars-BSA. This inhibition of protein phosphorylation correlated well with the inhibition of histamine and leukotriene release in bone marrow derived mast cells.

Mast cells and basophils are the primary target cells for immunoglobulin E (IgE)†, and the binding of multivalent antigens to the surface-bound IgE antibodies results in the release of a variety of inflammatory mediators [1, 2]. Alternatively, rat peritoneal mast cells can be activated via a receptor-mediated event using compound 48/80 [3]. Analysis of the biochemical events involved in mediator release revealed that the activation of various membrane-associated enzymes appears to be critically involved in the transduction of triggering signals for mediator release. Activation of both rat peritoneal and mouse bone marrow derived mast cells involves the modulation of a number of common biochemical mediators. Among these are increases in the level of cAMP [4–6] and intracellular Ca^{2+} [7], in the production of IP_3 and DAG [8], and in the translocation of protein kinase C from the cytosol to the plasma membrane [9]. All of these biochemical events take place within 1 min of receptor coupling and all precede mediator release. Previous results have suggested that the enzymes involved in the biochemical cascade may be different depending on stimuli [8].

Recent studies have revealed that protein kinase C may play a role in the signal transduction process in many of these reactions [10]. The purification [11, 12] of different isoenzymes of this enzyme has heightened speculations as to the role the various sub-species may play in signal transuction. However, the precise function of PKC in mast cell activation is uncertain. Experiments by ourselves and others have shown that activation of PKC with PMA leads to the translocation of protein kinase C from the cytosol to the plasma membrane [9, 13, 14].

In view of the multifaceted role protein kinase C plays in signal transduction, we decided to investigate the role PKC plays in mediator release in rat peritoneal and mouse bone marrow derived mast cells. Two PKC inhibitors, staurosporine and K-252a, isolated from *Streptomyces* sp. and *Nocardiopsis* sp.

* Correspondence: Dr. John R. White, Smith Kline & French Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939.

† Abbreviations: IgE, immunoglobulin E; PKC, protein kinase C; MBMDMC, mouse bone marrow derived mast cells; IL3, interleukin 3; PGE_2, prostaglandin E_2; LTC_4, leukotriene C_4; FCS, fetal calf serum; K-252a, (8*R**, 9*S**,11*S**)-(−)-9-hydroxyl-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11-atriazadibenzo-[a,g]cycloocta[cde]trinden-1-one; BSA, bovine serum albumin; PMA, phorbol,12-myristate,13-acetate; Ars-BSA, arsenate-bovine serum albumin; anti-Ars-IgE, mouse monoclonal anti-arsenate IgE; PS, phosphatidylserine; DAG, diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; cAMP, adenosine 3′-5 cyclic monophosphate; PT, pertusis toxin; IAP, islet activating protein; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; PMSF, phenylmethylsulfonlyl fluoride; and SDS, sodium dodecyl sulfate.

have been described recently [15–19]. Both are very potent inhibitors of protein kinase C with K_i values of 0.7 and 25 nM respectively. However, staurosporine and K-252a also inhibit cAMP-dependent protein kinase but to differing extents, with K_i values of 7 and 18 nM respectively. A number of studies using staurosporine and K-252a have shown that both inhibitors are capable of modulating cell activation in neutrophils [20] and platelets [21, 22]. The PKC antagonists staurosporine and K-252a were used to test the hypothesis that PKC is involved in the modulation of mediator release from mast cells. In this study we report results which suggest that PKC is involved in the transduction process resulting in mast cell degranulation stimulated by cross-linking of the IgE but not by the occupation of the 48/80 receptor.

MATERIALS AND METHODS

Cell preparation. Mouse bone marrow derived mast cells (MBMDMC) were obtained from a suspension culture of bone marrow cells of CBA/J mice (Jackson). The cells were cultured in RPMI 1640 culture medium (Gibco) supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1% each of non-essential amino acids, 10% WEHI III supernatant or 20 units/mL IL3 (Genzym, Boston, MA). After 4 weeks of culture, cells were >99% mast cells and >98% viable.

Purification of rat peritoneal mast cells. Rat mast cells were purified from male Sprague–Dawley (SD) rats (350–400 g, Charles River) which had been sensitized previously with two injections (i.p.) of egg albumin (0.01 μg/mL) in Maalox. After washing in Tyrode's buffer [pH 7.2, 124 mM NaCl, 4 mM KCl, 0.64 mM Na_2HPO_4, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (Hepes), 5 mM 4-morpholineethansulfonic acid (MES), 5.56 mM glucose and 1 mg/mL of bovine serum albumin], mixed peritoneal cells (2×10^7 cells in 2.5 mL) were layered over Percoll (Pharmacia) of the following volume and densities: 1.5 mL of 1.10ρ; 2 mL of 1.11ρ; 2 mL of 1.123ρ. The step gradients were then centrifuged (400 *g* for 20 min at 25°). Mast cells were found at the third interface and at the bottom of the test tube. Mast cells were recovered (60–80%) at greater than 98% purity. Cells purified by this method showed the same percent histamine release as their unpurified counterpart.

Inhibition of histamine release by PKC inhibitors K-252a and staurosporine. Mixed peritoneal cells containing between 1 and 5% mast cells were recovered from male Sprague–Dawley rats (350–400 g) by direct lavage and histamine secretion determined as previously described [23]. To examine the effect of PKC inhibitors on histamine release, mixed mast cells were incubated for 5 min with the appropriate drug before stimulation. After this preincubation, anti-IgE (1:300 dil) or compound 48/80 (1 μg/mL) was added, and histamine or PGE_2 release was assessed after a 10-min incubation. When anti-IgE was used, phosphatidylserine was added at a final concentration of 15 μg/mL, 5 min before stimulation.

Inhibition of leukotriene release by PKC inhibitors staurosporine and K-252a. To determine the effects of PKC inhibitors K-252a and staurosporine on leukotriene release from MBMDMC, the cells were sensitized with an optimal concentration of anti-Asc-IgE from clone HB137 (American Tissue Culture Collection, ATCC). After washing three times in complete Tyrode's buffer, cells were resuspended at 3×10^6 cells/mL and exposed to the appropriate concentration of the drug for 5 min before challenge with an optimal concentration of Ars-BSA (0.01 μg/mL). The release of immunoreactive leukotrienes, including leukotriene C_4, was assessed after a 15-min incubation. Cell supernatant fractions were stored at −70° until assayed using a commercial assay kit (New England Nuclear, Boston, MA). Drugs were prepared as 10^{-2} M stock solutions in DMSO. The final concentration of vehicle in the assay was less than 0.01%. Appropriate controls for vehicle effect were carried out in all experiments.

Inhibition of prostaglandin release from rat peritoneal mast cells by PKC inhibitors K-525a and staurosporine. Purified rat peritoneal mast cells were exposed to the two PKC inhibitors at the appropriate concentrations for 5 min before being stimulated by anti-IgE (1:300 dil) or compound 48/80 (1 μg/mL). When anti-IgE was employed, phosphatidylserine was dispersed in the medium 5 min before stimulation to a final concentration of 15 μg/mL. After a 10-min incubation, the cells were centrifuged (400 *g*, 10 min), and the supernatant fraction was decanted and assayed for PGE_2 production using a commercial assay kit (New England Nuclear).

Phosphorylation of substrate proteins in cell-free systems. To assess the phosphorylation of proteins in a cell-free system, 2×10^7 bone marrow derived mast cells were lysed by sonication in 500 μL of Ca^{2+}-free buffer (50 mM Tris–HCl, pH 6.5, 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 1 μM PMSF). The lysed cells (50 μL) were then exposed for phosphorylation in a mixture of 50 mM Tris–HCl, pH 7.5, containing 50 μM ATP (including 100 μCi [γ-^{32}P]ATP at 5000 Ci/mmol), 10 mM $MgCl_2$, 2 mM $CaCl_2$, phosphatidylserine at 40 μg/mL, and Diolein at 2 μg/mL in a total volume of 250 μL. After 5 min at 30°, 50 μL of the reaction mixture was withdrawn and added to 50 μL of 2× stock concentration of isoelectric focusing lysis buffer. To determine the calcium dependency of the kinase, a number of controls were included that omitted Ca^{2+} and/or phosphatidylserine/DAG. To test the effect of the two PKC inhibitors, various concentrations of the two inhibitors were added before [γ-^{32}P]ATP. Phosphorylated proteins were analyzed by two-dimensional SDS-gel electrophoresis.

Two-dimensional gel electrophoresis. To study phosphorylation of intracellular proteins, mouse bone marrow derived mast cells (40×10^6 cells at 2×10^6 cells/mL) were washed in phosphate-free RPMI 1640 medium with dialyzed FCS (10%) and WEHI III (10%) supernatant. Cells were then exposed overnight with 2–5 mCi of ^{32}P as orthophosphate and optimal concentrations of dialyzed anti-Ars-IgE. Cells were then washed three times in a regular phosphate containing Tyrode's buffer and 100 μL of cells exposed to drug or control (5 min) and then to agonist (0.01 μg/mL Ars-BSA) for 30 sec. To

stop the reaction, 50 μL of the cell suspension was withdrawn and added to lysis solution (9 M urea, 2% ampholine (pH 5–7; 0.4 mL/10 mL), ampholine (pH 3–10; 0.1 mL/10 mL), 5% β-mercaptoethanol, and 3% Nonidet P-40 (by vol.) The mixture (40 μL) was then applied to a 16-cm tube gel with ampholines running from pH 4–7 according to the method of O'Farrell [24]. The second dimension consisted of a 10% straight gel. After running, the gel was strained, destained and exposed for autoradiography using Kodak XAR-5 film at −70°. The intensity of phosphorylated proteins was assessed using a densitometer scanner (LKB).

V8 protease digest. To identify phosphoproteins from a broken cell preparation and those obtained from intact cells, V8 protease was used to obtain a fingerprint digestion. The method of Cleveland *et al.* [25] was used to identify the phosphoproteins. Briefly, after autoradiography of the second dimension and visualization of the 45K proteins, the appropriate phosphopeptides were excised and the polyacrylamide gel piece was allowed to expand in Tris buffer, pH 7.4, 1 mM EDTA and 10% glycerol (w/v). The extruded piece of gel was then digested in the stacking gel of a polyacrylamide gel for 30 min with 10–100 μg/lane of V8 protease (Calbiochem), and the digested polypeptides were resolved on a 15% gel which was poured with 1 mM EDTA. Phosphopeptides were visualized by autoradiography with Kodak XAR-5 film at −70°.

Reagents. Hepes, MES, compound 48/80, and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO), RPMI 1640 was purchased from Gibco and fetal calf serum from Hyclone (Denver, CO). Percoll was purchased from Pharmacia (Piscataway, NJ). PGE_2 and LTC_4 assay kits were purchased from New England Nuclear. Anti-Ars-IgE was prepared from clone HB137 available from the ATCC. [γ-^{32}P]ATP and [^{32}P]orthophosphate were purchased from Amersham (Arlington Heights, IL). Staurosporine and K-252a were purchased from Kyowa Hakko (Tokyo, Japan).

RESULTS

Effects of staurosporine and K-252a on histamine release and prostaglandin synthesis from rat peritoneal mast cells. The effects of the two PKC inhibitors staurosporine and K-252a were tested for their abilities to inhibit histamine release induced by anti-IgE (1:300 dil) and compound 48/80 (1 μg/mL). As shown in Fig. 1A, the two PKC antagonists completely inhibited histamine release induced by anti-

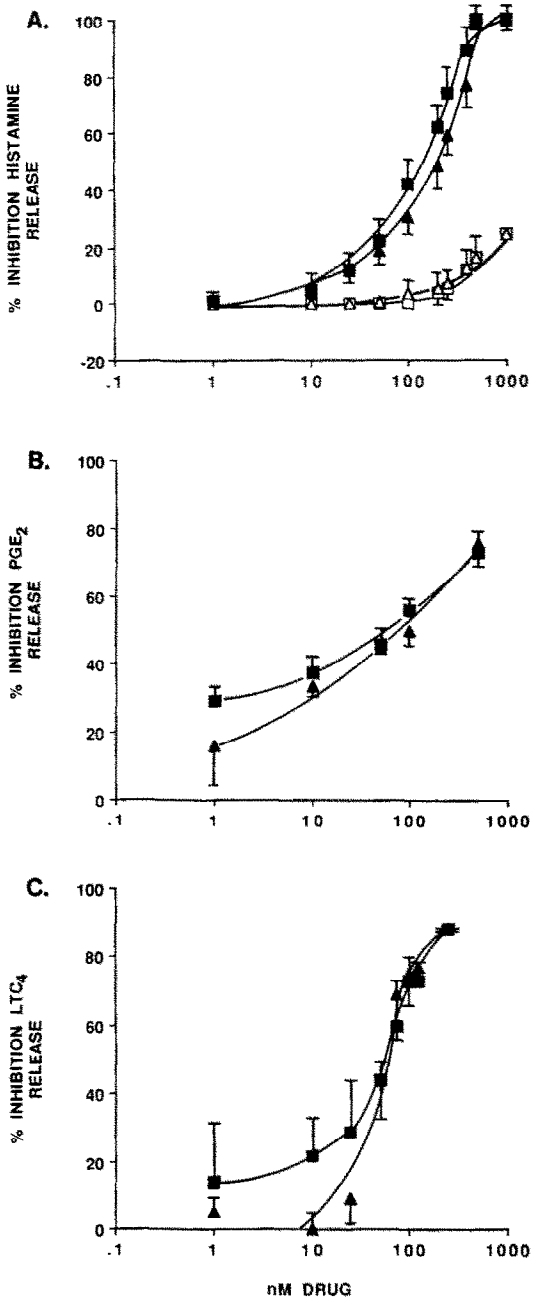

Fig. 1. Effects of various concentrations of the PKC inhibitors staurosporine (■, □) and K-252a (▲, △) on histamine, PGE_2 and LTC_4 release. (A) Histamine release from rat peritoneal mast cells. Cells obtained from Sprague–Dawley rats were treated with the appropriate concentration of drug for 5 min before being challenged with either anti-IgE (1:300 dil) (■, ▲) or compound 48/80 (1 μg/mL) (□, △). Histamine release was assessed after a further 15 min. Control histamine release was 8.5 ± 0.2 μg/10^6 mast cells (anti-IgE) or 9.9 ± 0.18 μg/10^6 mast cells with compound 48/80. (B) PGE_2 release from purified rat peritoneal mast cells. Rat mast cells were purified as described, and 1 × 10^6 mast cells with greater than 95% purity were exposed to anti-IgE (1:300 dil) (■, ▲). The reaction was allowed to proceed for 15 min before PGE_2 release was assessed. Control levels of PGE_2 were 70.3 ± 1.15 pg/10^6 cells. (C) Leukotriene C_4 release from mouse bone marrow derived mast cells. After sensitization with anti-Ars-IgE, cells were washed and incubated with staurosporine (■) or K-252a (▲) for 5 min before challenge with Ars-BSA (0.01 μg/mL). LTC_4 release in the absence of drug was found to be 27.0 ± 2.6 ng/10^6 cells. Each point in panels A, B and C is the mean ± SEM of four experiments.

IgE but failed to substantially inhibit histamine release induced by compound 48/80 even though both secretogogues led to the release of similar amounts of histamine (62.5 ± 4.9% for anti-IgE cf. 72.4 ± 2.1% for compound 48/80).

The release of PGE_2 from rat peritoneal mast cells stimulated with both anti-IgE and compound 48/80 was assessed. Anti-IgE induced the release of PGE_2 from purified rat peritoneal mast cells. In contrast, compound 48/80 failed to release significant amounts of the prostaglandin. Therefore, the inhibition of anti-IgE-induced PGE_2 synthesis by the two PKC inhibitors was evaluated. When purified rat peritoneal mast cells were stimulated with anti-IgE, both staurosporine and K-252a inhibited prostaglandin E_2 synthesis over the concentration–response range of 1 to 500 nM with IC_{50} values of 100 nM (Fig. 1B).

Effects of staurosporine and K-252a on histamine and leukotriene release from mouse bone marrow derived mast cells. Using MBMDMC as a model system, we investigated the effects of the two PKC inhibitors on both histamine and leukotriene C_4 release. MBMDMC were stimulated with Ars-BSA (0.01 μg/mL) to which they had been sensitized by overnight incubation with optimal concentrations of IgE (anti-Ars) antibody. As can be seen from Fig. 1C, both staurosporine and K-252a inhibited LTC_4 release in a concentration-dependent manner. Staurosporine inhibited LTC_4 release with an identical IC_{50} as K-252a (50 nM). The two analogs also inhibited histamine (IC_{50} = 50 nM) release (see Fig. 6) when sensitized mouse bone marrow derived mast cells were stimulated with an optimal concentration of Ars-BSA.

Identification of PKC substrate proteins. Because the mode of action of protein kinase C is believed to be activation/deactivation of enzymatic activity by phosphorylation of intracellular substrate proteins, we investigated the effect of PKC activators (i.e. PMA, Ars-BSA and Ca^{2+}/phosphatidylserine/Diolein) on the phosphorylation of intracellular proteins by protein kinase C in both whole and broken cell preparations. As shown in Fig. 2, treatment of mouse bone marrow derived mast cells with Ars-BSA led to the selective phosphorylation of three proteins of 45K molecular weight. These three phosphoproteins were the major proteins phosphorylated upon cell activation by Ars-BSA. To characterize these proteins as specific substrates for PKC, broken cell preparations were treated with [γ-^{32}P]ATP in the presence or absence of Ca^{2+} and/or phosphatidylserine/Diolein as control. Analysis of the phosphorylated proteins showed that the three 45K proteins of pI 6.2–6.5 were phosphorylated only under conditions where protein kinase C is active, i.e. in the presence of both Ca^{2+} and phosphatidylserine (Fig. 3). Treatment of the broken cell preparation with Ca^{2+} alone led to the phosphorylation of a number of other proteins. However, this phosphorylation was not PS dependent and, therefore, unlikely to be a PKC-mediated event. Furthermore, treatment of this broken cell preparation with either of the two PKC inhibitors (50–500 nM) led to a specific and equal concentration-related reduction in the phosphate content of the two specific PKC substrate proteins (results not shown).

Treatment of intact ^{32}P-labeled mast cells with PMA (50 ng/mL) resulted in the phosphorylation of the same 45K proteins (results not shown). To determine if these proteins were the same under the three stimulatory conditions, intact cells were stimulated with Ars-BSA or PMA or broken cells were stimulated with PS/Diolein and Ca^{2+} in the presence of [γ-^{32}P]ATP, and the 45K phosphoproteins were extracted from the resulting two-dimensional SDS-gel and digested by V8 protease (Cleveland digestion). The resulting phosphopeptides were analyzed by single-dimension SDS-gel electrophoresis. Cleveland digestion of the 45K phosphoproteins (proteins 1, 2, and 3) from the whole cell resulted in two phosphopeptide digestion products of 24K and 26K (phosphoprotein 2 is shown in Fig. 4, lane A; digestion of 1 and 3 are not shown). Digestion of phosphoproteins 1 and 3 from the broken cell preparation (lanes C and D, Fig. 4) resulted in two digestive products also of 24K and 26K that were identical to the products produced by Cleveland digestion for the whole cell 45K phosphoproteins. Analysis of the three phosphoproteins produced by PMA stimulation showed that all three of these proteins also produced two phosphopeptides upon treatment with V8 protease, one of which is shown in Fig. 4, lane F. For comparison, two unrelated phosphoproteins (lanes E and B) located at molecular weights of 66 and 100K and both having pI values of 6 were used as controls. These two control phosphoproteins were obtained from phosphorylated whole and lysed cells respectively. Neither of the control proteins showed a digestive pattern similar to the 45K phosphoproteins.

Inhibition of 45K protein phosphorylation by K-252a and staurosporine. To determine if either of the two microbial alkaloids can inhibit the 45K protein phosphorylation, ^{32}P-labeled mouse bone marrow derived mast cells were stimulated with Ars-BSA in the presence or absence of the two inhibitors. Figure 5 shows that both compounds led to the selective inhibition of the 45K phosphoproteins. The middle arrow (protein 2, Fig. 5) identifies the 45K protein which was most susceptible, being inhibited almost completely by K-252a and staurosporine at 500 nM. The right arrow (protein 3, Fig. 5) shows the next phosphoprotein which also was inhibited significantly, but a higher concentration of both inhibitors was required for complete inhibition. The phosphoprotein (protein 1) was only inhibited significantly by staurosporine (500–1000 nM); K-252a failed to show any inhibition. Comparison of the curve for inhibition of protein phosphorylation (proteins 1, 2 and 3) showed that only the center phosphoprotein correlated well with the curve for inhibition of mediator release (Fig. 6). The other two phosphoproteins (proteins 1 and 3) required higher concentrations of the microbial alkaloids than were required for the inhibition of mediator release (results not shown).

DISCUSSION

Previous studies by several investigators have shown that cross-linking of surface bound IgE on mast cells leads to a number of biochemical events

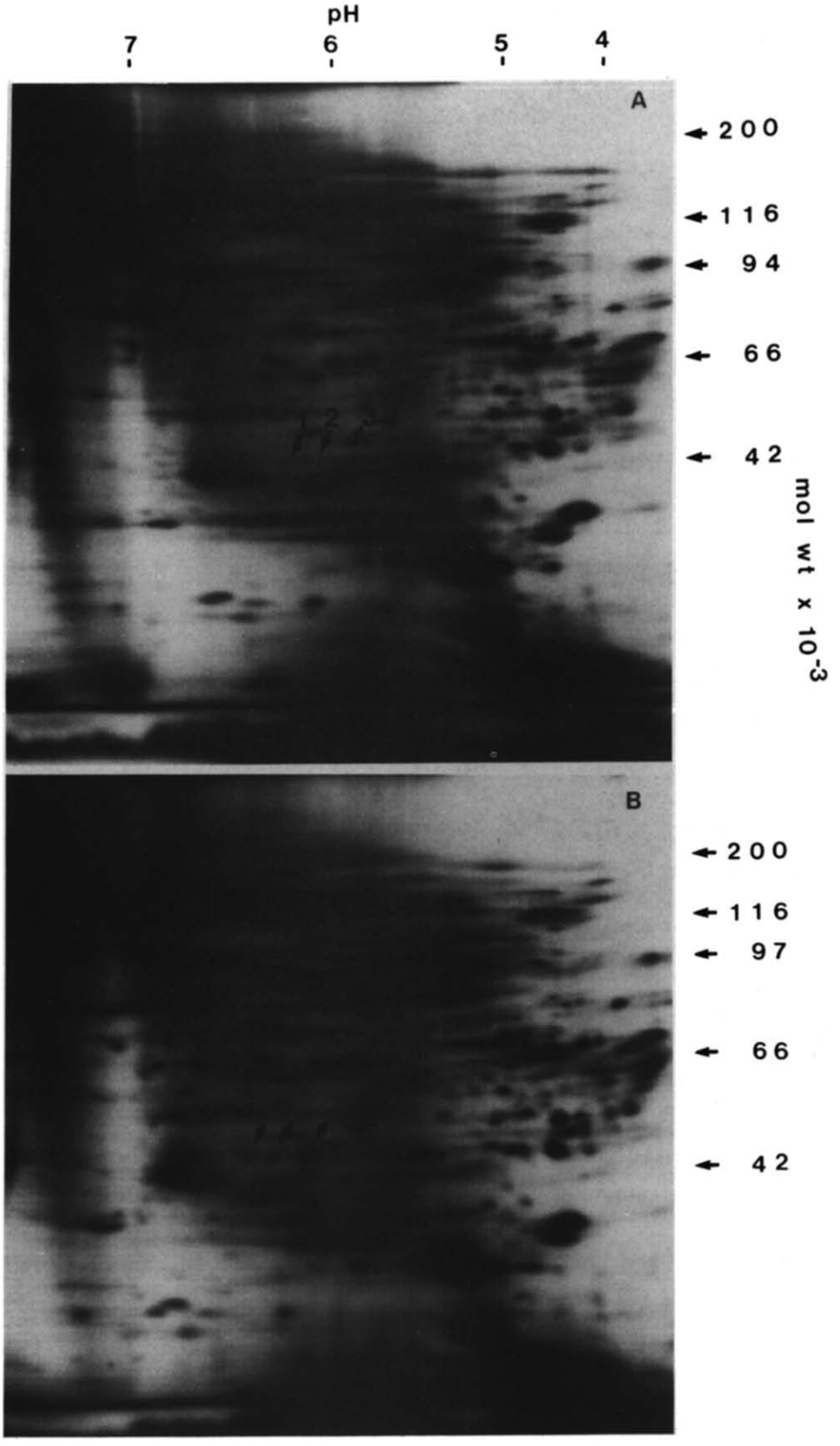

Fig. 2. Effect of Ars-BSA on protein phosphorylation in mouse bone marrow derived mast cells. Cells were pre-labeled with [^{32}P]orthophosphate (2–5 mCi) overnight in phosphate-free RPMI 1640, dialyzed WEHI III and anti-Ars-IgE antibody. Cells were then treated with Ars-BSA (0.01 μg/mL) for 30 sec. Thereafter, the cells were lysed and the whole cell protein was resolved by two-dimensional gel electrophoresis. (A) control. (B) Ars-BSA. Arrows indicate the three 45K phosphoproteins.

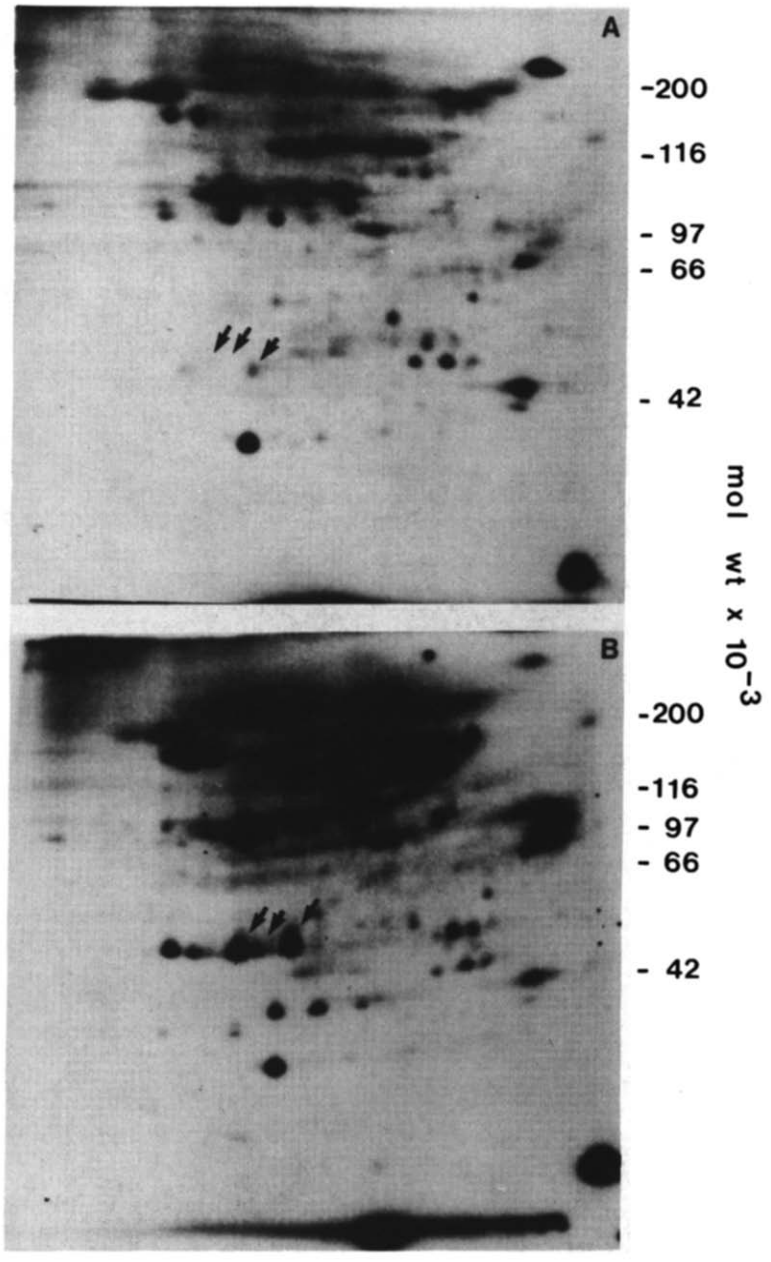

Fig. 3. Phosphorylation of proteins in broken cell preparation. Mouse bone marrow derived mast cells were lysed by sonication, and the particulate matter was removed by centrifugation (100,000 *g*, 60 min). The cytosol was then exposed to [γ^{32}P]ATP with or without cofactors. (A) Control, no additions. (B) Additions included: Ca^{2+} and phosphatidylserine/Diolein. The phosphorylation proceeded for 5 min (30°) before the proteins were resolved by two-dimensional gel electrophoresis.

[4–6, 8]. Among these are a transient increase in the level of cAMP, an increased turnover of phosphoinositides and a concomitant production of both IP_3 [4–6] and DAG [8]. It has been proposed that IP_3 is responsible for the release of Ca^{2+} from intracellular stores, notably the endoplasmic reticulum [26]. Diacylglycerol has been shown to activate protein kinase C and may participate in the translocation of the kinase from the cytosol to the cell membrane [27]. This translocation is accompanied by specific phosphorylation of intracellular proteins thought to be essential participants in cell activation [28].

Recently, Saito *et al.* [8] demonstrated a divergence in the pathways employed by at least two of the receptors on the surface of the mast cell. Using pertussis toxin (PT), they found that up to 97% of

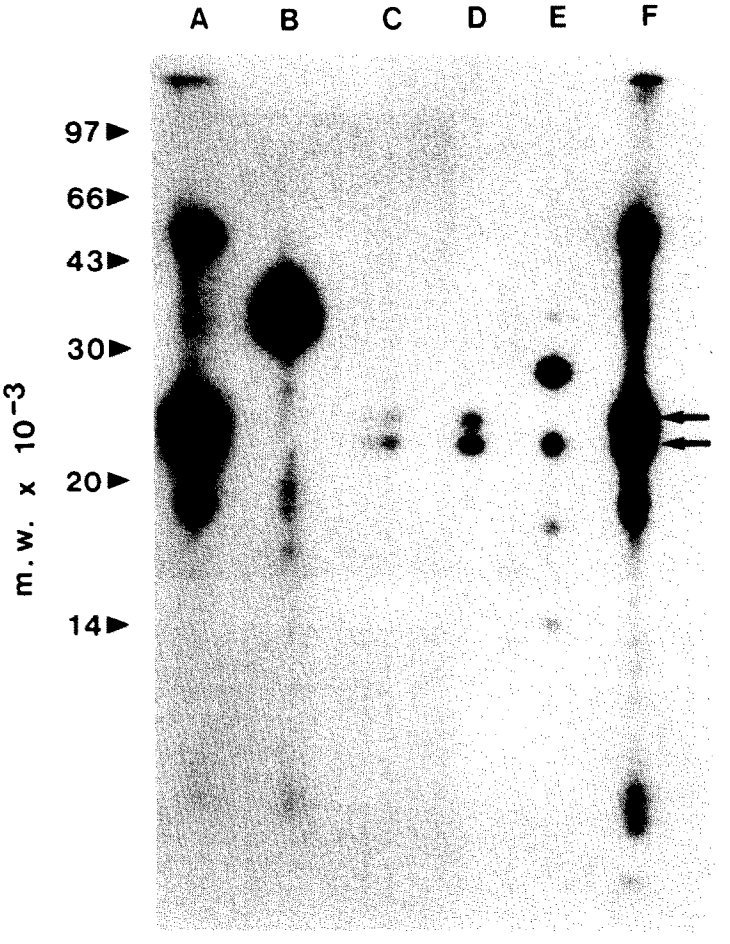

Fig. 4. Cleveland digestion of phosphoproteins. Lane A: whole cells stimulated with Ars-BSA (30 sec). The 45K phosphoprotein (protein 2) was digested with 10 μg/lane of V8 protease. Lane B: 100K control protein from stimulated whole cell. Lanes C and D: broken cell preparations were stimulated in the presence of Ca^{2+} and PS/Diolein with [γ-^{32}P]ATP for 5 min at 30°. Lane C shows the digestion product of protein 1 and lane D, the digestion product of protein 3. Lane E: control 66K protein from broken cell preparation. Lane F: whole cells stimulated with PMA (50 ng/mL, 5 min). Protein 1 was excised and digested with V8 protease. Arrows indicate 24K and 26K phosphoproteins.

the 41K G-protein had been ADP-ribosylated after treatment with optimal concentrations of PT. Stimulation of the cells via two independent surface receptors, namely anti-IgE and compound 48/80 in the presence of PT, led to the selective inhibition of histamine release elicited by compound 48/80 but failed to inhibit anti-IgE mediated degranulation. This observation suggests that either IgE does not utilize a G-protein for signal transduction or that the G-protein is a novel, non-IAP sensitive isotype [29]. We therefore carried out a number of experiments to determine if there were any more biochemical pathways which might be unique to one of the receptor classes.

Stimulation of rat peritoneal mast cells with anti-IgE leads to the release of histamine and the synthesis of prostaglandin E_2, whereas activation of these same cells with 48/80 leads to the release of histamine without the synthesis of PGE_2. Using the two PKC inhibitors, we evaluated the potential for inhibiting mediator release induced by these two secretagogues. When rat peritoneal mast cells were stimulated with anti-IgE, both PKC inhibitors prevented histamine release and the synthesis of prostaglandin E_2. When the same cells were stimulated with compound 48/80, both inhibitors failed to produce a significant inhibition of histamine release. Previously we demonstrated that antigen-induced histamine release in PT-18 cells is associated with a rapid increase in PKC activity associated with the plasma membrane [9]. Here we present more evidence that PKC plays a crucial role in mediating IgE-dependent mediator release. The failure of either of these two PKC inhibitors to modulate substantially 48/80-induced degranulation suggests that either the 48/80 receptor is not linked to PKC as an intracellular modulator or that the isotype of PKC used by 48/80 is not susceptible to inhibition by either of these two inhibitors.

To confirm that the two PKC inhibitors were inhi-

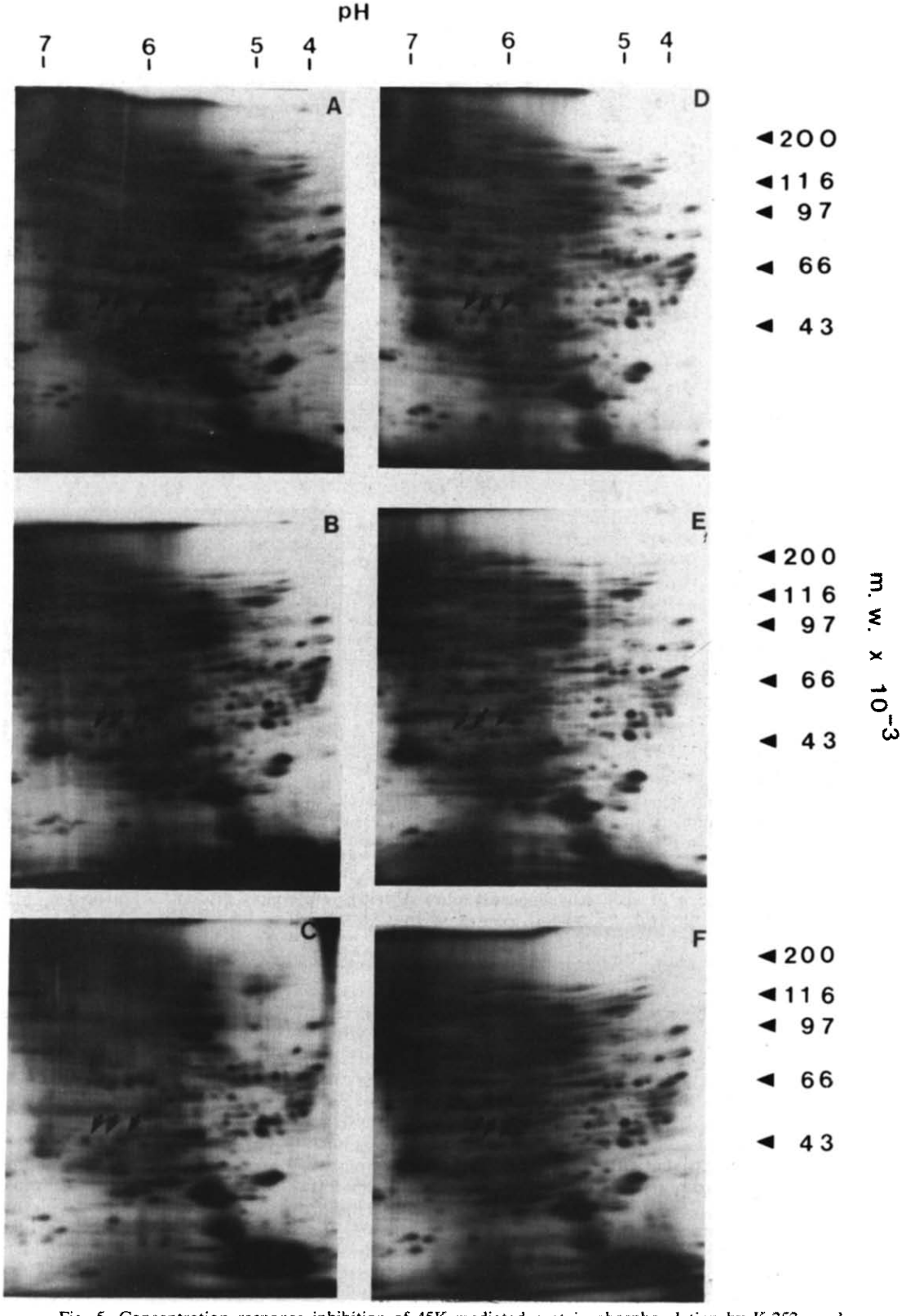

Fig. 5. Concentration–response inhibition of 45K mediated protein phosphorylation by K-252a and staurosporine. Mouse bone marrow derived mast cells were incubated with the respective inhibitor for 5 min prior to Ars-BSA stimulation, which was allowed to proceed for 30 sec before the cells were lysed and the total cell protein was analyzed by two-dimensional gel electrophoresis. The three arrows indicate the 45K protein which was phosphorylated by PKC. Control cells ± Ars-BSA are shown in Fig. 2. Panel key: (A) K-252a, 100 nM; (B) K-252a, 500 nM; (C) K-252a, 1000 nM; (D) staurosporine, 100 nM; (E) staurosporine, 500 nM; and (F) staurosporine, 1000 nM.

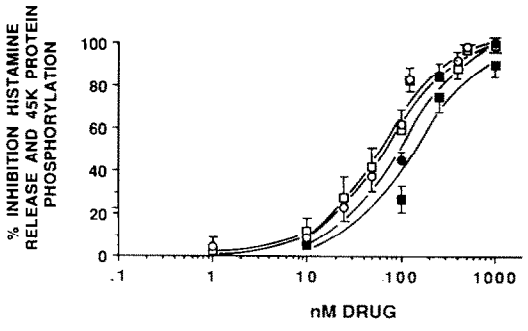

Fig. 6. Effects of various concentrations of staurosporine and K-252a on histamine release (open symbols) and 45K protein phosphorylation (protein 2, closed symbols) in mouse bone marrow derived mast cells. MBMDMC were sensitized overnight and either labeled with ^{32}P as orthophosphate (for phosphorylation) or cultured in RPMI 1640 medium (for histamine release). For histamine release, cells were resuspended at 2×10^6 cells/mL and after a 5-min preincubation with staurosporine (○) or K-252a (□) stimulated with Ars-BSA (0.01 μg/mL) for 10 min. Control histamine release was 262 ± 18 ng/10^6 mast cells. For protein phosphorylation, ^{32}P-labeled MBMDMC were incubated with staurosporine (●) or K-252a (■) for 5 min before stimulation with Ars-BSA (0.01 μg/mL). Protein phosphorylation was assessed after a 30-sec stimulation. Each histamine point is the mean ± SE of four experiments; each protein phosphorylation point is the mean ± SE of three experiments.

biting mediator release through the modulation of a PKC-dependent protein phosphorylation, a PKC substrate protein was identified in the cytosol of mouse bone marrow derived mast cells. Cross-linking of IgE by Ars-BSA or the direct activation of protein kinase C by PMA led to a rapid phosphorylation of three proteins with molecular weights of 45K and pI values between 6 and 6.5. These proteins appeared to be different from those reported by Katakami *et al.* [30] on the basis of their molecular weights. Other proteins were phosphorylated under the conditions mentioned above, but they were minor and difficult to quantitate. These 45K proteins were also phosphorylated in a broken cell preparation but only in the presence of Ca^{2+} and phosphatidylserine/Diolein. Calcium or phosphatidylserine/Diolein alone failed to cause phosphorylation, indicating that these three proteins are selective substrate proteins for protein kinase C phosphorylation. However, Ca^{2+} alone did lead to the phosphorylation of a number of other proteins, indicating that their phosphorylation is a Ca^{2+}-dependent event and does not involve PKC. To confirm that the three 45K phosphoproteins phosphorylated under the conditions mentioned above are related, each band was digested with V8 protease, and the peptides were analyzed. The peptide map, revealed after Cleveland digestion, suggested that the three 45K phosphoproteins are not only related between the three conditions of activation, namely Ars-BSA, PMA, and in the broken cell preparation by Ca^{2+} and PS/Diolein, but they are also related to each other. The most likely explanation for their change in pI value is a post-translational modification where there is a sequential addition of a phosphate group for each of the shifts in pI value.

Pretreatment of mouse bone marrow derived mast cells with either of the two PKC inhibitors before stimulation with Ars-BSA or PMA resulted in a concentration-related inhibition in the phosphate content of the 45K phosphoproteins. Analysis by optical density of the three phosphoproteins indicated that the middle phosphoprotein was the most susceptible to inhibition by both inhibitors. A comparison of the concentration–response curve for the inhibition of mediator release with that for protein phosphorylation indicated that the middle phosphoprotein was inhibited to the same extent and over the same concentration–response curve as mediator release. In comparison, the other two phosphoproteins (1 and 3) were not inhibited so dramatically. A comparison of inhibition of mediator release with that for the inhibition of protein phosphorylation showed that higher concentrations of both inhibitors were required to inhibit protein phosphorylation than mediator release. The failure of proteins 1 and 3 to be inhibited completely by either of the two PKC inhibitors even though this phosphorylation appears to be PKC mediated may be explained in part by (1) the fact that there is a difference in time between stimulation and the analysis for protein phosphorylation (30 sec) and mediator release (10 min), (2) the degree to which protein phosphorylation is arrested may only have to be small to effect a large inhibition of mediator release, (3) a different isotype of PKC is employed in the phosphorylation of proteins 1 and 3 which is not as susceptible to inhibition by the two PKC inhibitors as the PKC isotype performing the phosphorylation of protein 2, or (4) Cunha-Melo *et al.* [31] recently showed that staurosporine is capable of inhibiting the production of inositol phosphates in RBL-2H3 basophils. Such an hypothesis applied to our results could offer an alternative explanation for the inhibition of mediator release observed in rat peritoneal mast cells. However, this rational is unlikely because compound 48/80 mediated degranulation was not inhibited to the same extent as IgE mediated degranulation (Fig. 1A). Yet compound 48/80 produces a significant increase in Ca^{2+} [6] and DAG [32] and the metabolism of inositol phosphates [32], all of which, at least in part, are required for both IgE and 48/80-dependent mediator release.

Activation of rat peritoneal or mouse bone marrow derived mast cells by aggregation of surface IgE molecules results in specific PKC-dependent protein phosphorylation. Previous results have suggested that the signal transduced by IgE aggregation does not utilize a G-protein or uses an IAP-insensitive form [29]. Results presented in this paper show that PKC inhibitors prevented IgE mediated histamine and leukotriene release. On the other hand, compound 48/80 which is IAP sensitive was not inhibited to the same extent, suggesting that 48/80 does not utilize PKC as a secondary signal but rather uses G-proteins which modulate mediator release independently of PKC. It would thus appear that IgE and 48/80 use different signal transduction mechanisms in promoting histamine and other mediator release.

REFERENCES

1. Ishizaka T, Analysis of triggering events in mast cells for immunoglobulin E-mediated release. *J Allergy Clin Immunol* **67**: 90–96, 1981.
2. Sullivan TJ and Parker CW, Pharmacological modulation of inflammatory mediator release by rat mast cells. *Am J Pathol* **85**: 437–464, 1976.
3. Hino RH, Lau CKH and Read GW, The site of action of the histamine releaser compound 48/80 in causing mast cell degranulation. *J Pharmacol Exp Ther* **200**: 658–663, 1977.
4. Sullivan TJ, Parker KL, Kulczycki A and Parker CW, Modulation of cyclic AMP in purified rat mast cells. III. Studies on the effect of concanavalin A and anti-IgE on cAMP concentrations during histamine release. *J Immunol* **117**: 713–716, 1976.
5. Lewis RA, Holgate ST, Roberts JJ, Maguire JF, Oates JA and Austen KF, Effect of indomethacin on cyclic nucleotide levels and histamine release from rat serosal mast cells. *J Immunol* **123**: 1663–1668, 1979.
6. Lichtenstein LM, Sobotka AK, Malveaux HJ and Gillespie E, IgE-induced changes in human basophil cyclic AMP levels. *Int Arch Allergy Appl Immunol* **56**: 473–478, 1978.
7. White JR, Ishizaka T, Ishizaka K and Sha'afi RI, Direct demonstration of increased intracellular concentration of free calcium as measured by quin-2 in stimulated rat peritoneal mast cells. *Proc Natl Acad Sci USA* **81**: 3978–3982, 1984.
8. Saito H, Okajima F, Moiski TFP, Sha'afi RI, Ui M and Ishizaka T, Effect of ADP-ribosylation of GTP-binding protein by pertussis toxin on immunoglobulin E-dependent and -independent histamine release from mast cells and basophils. *J Immunol* **138**: 3927–3934, 1987.
9. White JR, Pluznik DH, Ishizaka K and Ishizaka T, Antigen-induced increase in protein kinase C activity in plasma membrane of mast cells. *Proc Natl Acad Sci USA* **82**: 8193–8197, 1984.
10. Kraft AS, Andersen WB, Cooper HL and Sando JJ, Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. *J Biol Chem* **257**: 13193–13196, 1982.
11. Huang KP, Nakabayashi H and Huang FL, Isozymic forms of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase. *Proc Natl Acad Sci USA* **83**: 8535–8539, 1986.
12. Woodgett JR and Hunter T, Immunological evidence for two physiological forms of protein kinase C. *Mol Cell Biol* **7**: 85–96, 1987.
13. Erne P, Mazurek N and Borner C, Translocation of protein kinase C is not required to inhibit the antigen-induced increase of cytosolic calcium in mast cell line. *Biochem Biophys Res Commun* **143**: 252–259, 1987.
14. Nagao S, Nagata K, Kohmura Y, Ishizuka T and Nozawa Y, Redistribution of phospholipid/Ca^{2+}-dependent protein kinase in mast cells activated by various agonists. *Biochem Biophys Res Commun* **142**: 645–653, 1987.
15. Kase H, Iwahashi K and Matsuda Y, K-252a, A potent inhibitor of protein kinase C from microbial origin. *J Antibiot (Tokyo)* **39**: 1059–1065, 1986.
16. Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C and Sato A, K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun* **142**: 436–440, 1987.
17. Nakanishi S, Matsuda Y, Iwahashi K and Kase H, K-252b, c and d, potent inhibitors of protein kinase C from microbial origin. *J Antibiot (Tokyo)* **39**: 1066–1071, 1986.
18. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M and Tomita F, Staurosporine, a potent inhibitor of phospholipid/Ca^{++}-dependent protein kinase. *Biochem Biophys Res Commun* **135**: 397–402, 1986.
19. Yasuzawa T, Iida T, Yoshida M, Hirayama N, Takahashi M, Shirahata K and Sano H, The structures of the novel protein kinase C inhibitors K-252a, b, c and d. *J Antibiot (Tokyo)* **39**: 1072–1078, 1986.
20. Smith RJ, Justen JM and Smith LM, Effects of a protein kinase C inhibitor, K-252a, on human polymorphonuclear neutrophil responsiveness. *Biochem Biophys Res Commun* **152**: 1497–1503, 1988.
21. Yamada K, Kazuyuki I and Kase H, K252a, a new inhibitor of protein kinase C, concomitantly inhibits 40K protein phosphorylation and serotonin secretion in a phorbol ester-stimulated platelets. *Biochem Biophys Res Commun* **144**: 35–40, 1987.
22. Schächtele C, Seifert R and Osswald H, Stimulus-dependent inhibition of platelet aggregation by the protein kinase inhibitors polymixin B, H-7 and staurosporine. *Biochem Biophys Res Commun* **151**: 542–547, 1988.
23. White JR and Pearce FL, Effect of anti-allergic compounds on anaphylactic histamine release from rat peritoneal mast cells in the presence and absence of exogenous calcium. *Immunology* **46**: 361–367, 1982.
24. O'Farrell PH, High resolution two-dimensional electrophoresis proteins. *J Biol Chem* **250**: 4007–4021, 1975.
25. Cleveland DW, Fischer SG, Kirschner MW and Laemmli UK, Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J Biol Chem* **252**: 1102–1106, 1977.
26. Streb HP, Irvine RF, Berridge MJ and Schultz I, Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-triphosphate. *Nature* **306**: 67–69, 1983.
27. Kishimoto A, Takai Y, Mori U, Kikkawa T and Nishizuka YJ, Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J Biol Chem* **255**: 2273–2276, 1980.
28. Connolly TM, Lawing WJ and Majerus PW, Protein kinase C phosphorylates human platelet inositol triphosphate 5'-phosphomonoesterase increasing the phosphatase activity. *Cell* **46**: 951–958, 1986.
29. Matsuoka M, Itoh H, Kozasa T and Kaziro Y, Sequence analysis of cDNA and genomic DNA for a putative pertussis toxin-sensitive guanine nucleotide-binding regulatory protein subunit. *Proc Natl Acad Sci USA* **85**: 5384–5388, 1988.
30. Katakami Y, Kaibuchi K, Sawamura M, Takai Y and Nishizuka Y, Synergistic action of protein kinase C and calcium for histamine release from rat peritoneal mast cells. *Biochem Biophys Res Commun* **121**: 573–578, 1984.
31. Cunha-Melo J, Gonzaga HMS, Ali H, Huang FL, Huang K-P and Beaven MA, Studies of protein kinase C in the rat basophilic leukemia (RBL-2H3) cell reveal that antigen-induced signals are not mimicked by the actions of phorbol myristate acetate and Ca^{2+} ionophore. *J Immunol* **143**: 2617–2625, 1989.
32. Dainaka J, Ichikawa A, Koibuchi Y, Nakagawa M and Tomita K, Effect of the tridecamer of compound 48/80, a Ca^{2+}-dependent histamine releaser, on phospholipid metabolism during the early stage of histamine release from rat mast cells. *Biochem Pharmacol* **35**: 3739–3744, 1986.